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Research report

# Valproate pretreatment protects dopaminergic neurons from LPS-induced neurotoxicity in rat primary midbrain cultures: role of microglia

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**Abstract**

Parkinson's disease is a neurodegenerative disorder characterized by progressive degeneration of dopaminergic (DA) neurons in the substantia nigra. Accumulating evidence supports the notion that neuroinflammation is involved in the pathogenesis of this disease. Valproate (VPA) has long been used for the treatment of seizures and bipolar mood disorder. In vivo and in vitro studies have demonstrated that VPA has neuroprotective and neurotrophic actions. In this study, using primary neuron–glia cultures from rat midbrain, we demonstrated that VPA is a potent neuroprotective agent against lipopolysaccharide (LPS)-induced neurotoxicity. Results showed that pretreatment with 0.6 mM VPA for 48 h robustly attenuated LPS-induced degeneration of dopaminergic neurons as determined by [$^3$H] dopamine uptake and counting of the number of TH-ir neurons. The neuroprotective effect of VPA was concentration-dependent and was mediated, at least in part, through a decrease in levels of pro-inflammatory factors released from activated microglia. Specifically, LPS-induced increase in the release of TNFa, NO, and intracellular reactive oxygen species was markedly reduced in cultures pretreated with VPA. These anti-inflammatory effects of VPA were time and concentration-dependent correlated with a decrease in the number of microglia. Thus, our results demonstrate that protracted VPA pretreatment protects dopaminergic neurons from LPS-induced neurotoxicity through a reduction in levels of released pro-inflammatory factors, and further suggest that these anti-inflammatory effects may be contributed by VPA-induced reduction of microglia cell number. Taken together, our study reinforces the view that VPA may have utility in treating Parkinson's disease.


*Keywords:* Parkinson's disease; Valproic acid; Microglia; Neuroprotection; Anti-inflammation

## 1. Introduction

Parkinson's disease is an age-related neurodegenerative disorder characterized by progressive degeneration of dopaminergic (DA) neurons within the substantia nigra, which leads to movement disorders including resting tremor, slowness of movement, rigidity, and postural instability. The etiology as well as the precise mechanisms underlying the selective destruction of the nigrostriatal dopaminergic pathway remains elusive. Recently, evidence from clinical [28,41] and animal studies [6,10,14,19,25,26,30,48]

*Abbreviations:* CM-H2-DCFDA, 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DA, dopamine; DCF, 2′-7′-dichlorofluorescein; ERK, extracellular signal related kinase; GAP-43, growth associated protein-43; HBSS, Hank's balanced salt solution; IL-6, interleukin-6; iROS, intracellular reactive oxygen species; KRB, Krebs–Ringer buffer; LPS, lipopolysaccharide; MEM, minimal essential medium; NO, nitric oxide; PD, Parkinson's disease; SOD, superoxide dismutase; TH-ir, tyrosine hydroxylase-immunoreactive; TNF-α, tumor necrosis factor-alpha; VPA, valproate

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suggests the involvement of neuroinflammation in the pathogenesis of Parkinson's disease. Additionally, a number of studies demonstrate that inhibition of the inflammatory reaction attenuates degeneration of nigrostriatal dopamine-containing neurons in several models of Parkinson's disease [18,35,50]. Inflammation-related neurodegeneration in the central nervous system is primarily mediated through the activation of microglial cells and the production of pro-inflammatory and cytotoxic factors. Microglia, the resident immune cells of the brain, serve the role of immune surveillance and host defense [26]. They are sensitive to infection or changes in their microenvironments and readily become activated in response to infection or injury. Activated microglia secrete a variety of factors, including cytokines, tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), free radicals, nitric oxide and superoxide, fatty acid metabolites, eicosanoids, and quinolinic acid [32]. Excessive quantities of individual pro-inflammatory and cytotoxic factors produced by activated microglia are deleterious to neurons [3,8,21,38,42]. Moreover, these individual factors can work in synergy to aggravate neurodegeneration [9,21]. The substantia nigra region of the brain is rich in microglia [25]. Over-activation of nigral microglia and release of excessive neurotoxic factors may be a risk factor in triggering the onset of a cascade of events leading to a progressive degeneration of DA neurons.

Valproate (VPA), a simple eight-carbon branched-chain fatty acid, is widely used for the treatment of seizures and as a mood stabilizer for treating bipolar disorder [23,49]. Clinical studies have revealed that VPA could relieve marked end-stage-Parkinsonism-rigidity in a small population of patients, while another patient population receiving VPA experienced reversible Parkinsonism and cognitive impairment [2,44]. Moreover, growing evidence from primary neuronal cultures and cell lines has demonstrated that VPA has neuroprotective effects against neural apoptosis [24,29,43], neurotrophic effects by activating the ERK pathway [12] and neuroplasticity roles by increasing the expression of growth associated protein GAP-43 [51] and neurite out-growth [22,51]. In addition, VPA has been shown to have anti-inflammatory effects by attenuating the production of TNFα and IL-6 in a human monocytic leukemia cell line through the inhibition of NF-κB activation in a human glioma cell line [20]. Although clinical use of VPA for Parkinson's disease has been reported, mechanisms of microglia involved in the protection of DA neurons by VPA has not been studied.

In this study, we used primary neuron–glia cultures prepared from rat embryonic midbrain, and stimulated with LPS to produce specific DA neuronal degeneration. The goals of the present study are to use the above model of inflammation-mediated DA neurodegeneration to: (1) determine whether VPA protects DA neurons from LPS-induced neurotoxicity through the inhibition of microglial activation; (2) characterize the anti-inflammatory responses of cultures stimulated with LPS and pretreated with VPA.

## 2. Materials and methods

### 2.1. Reagents

VPA was purchased from Sigma-Aldrich (St. Louis, MO). Cell culturing materials were obtained from Invitrogen (Carlsbad, CA). [$^3$H] DA (30 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA). The monoclonal antibody against the CR3 complement receptor (OX-42) was obtained from BD PharMingen (San Diego, CA). The polyclonal anti-tyrosine hydroxylase (TH) antibody was a generous gift from Dr. John Reinhard (GlaxoSmithKline, Research Triangle Park, NC). The Vectastain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). The CyQUANT cell proliferation assay kit was purchased from Molecular Probes (Eugene, OR).

### 2.2. Animals

Timed-pregnant Fisher F344 rats were obtained from Charles River Laboratories (Raleigh, NC). Housing and breeding of the animals were performed in strict accordance with the guidelines of the National Institutes of Health.

### 2.3. Primary mesencephalic neuron–glia cultures

Neuron–glia cultures were prepared from the ventral mesencephalic tissues of embryonic day 13–14 rats, as described previously [31,32]. Briefly, dissociated cells were seeded at $1\times10^5$/well and $5\times10^5$/well to poly-D-lysine-coated 96-well and 24-well plates, respectively. Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air, in minimal essential medium (MEM) containing 10% fetal bovine serum, 10% horse serum, 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin. Six days after seeding, the medium was changed to 2% MEM (2% fetal bovine and horse serum in MEM) and the cultures were then pretreated with vehicle or the indicated concentration (0.05, 0.2, 0.4 and 0.6 mM) of VPA, which was freshly prepared with culture medium. Two days later, cells were treated with 20 ng/ml LPS for 3 days and followed by the assay of [$^3$H] DA uptake or immunostaining.

### 2.4. Microglia-enriched cultures

Microglia was prepared from the whole brains of 1-day-old rats as described previously [36]. Immunocytochemical analysis indicated that the cultures were 95–98% pure for microglia.

### 2.5. Assessment of neurotoxicity

Degeneration of DA neurons was assessed by measuring the ability of cultures to take up [$^3$H] DA, or counting the

number of TH-ir neurons after immunostaining (see below) [40]. [$^3$H] DA uptake assays were performed as previously described [34]. Briefly, after washing twice with warm Krebs–Ringer buffer (KRB, 16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4), cultures were incubated for 20 min at 37 °C with 1 μM [$^3$H] DA in KRB for DA uptake. Afterwards, cultures were washed three times with ice-cold KRB and cells were then dissolved in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Nonspecific DA uptake determined in the presence of mazindol (10 μM) was subtracted.

### 2.6. Immunostaining

DA neurons were recognized with the anti-TH antibody and microglia were detected with the OX-42 antibody, which recognizes the CR3 receptor as described previously [34]. Briefly, formaldehyde (3.7%)-fixed cultures were treated with 1% hydrogen peroxide (10 ml) followed by sequential incubation with blocking solution (30 min), primary antibody (overnight, 4 °C), biotinylated secondary antibody (2 h), and ABC reagents (40 min). Color was developed with 3,3′-diaminobenzidine. For morphological analysis, the images were recorded with an inverted microscope (Nikon, Tokyo, Japan) connected to a charge-coupled device camera (DAGE-MTI, Michigan City, IN) operated with MetaMorph software (Universal Imaging, Downingtown, PA). TH-ir neurons in each well of the 24-well plate were visually counted under the microscope at 400× magnification.

### 2.7. TNFα and nitrite assays

The release of TNFα was measured with a rat TNFα enzyme-linked immunosorbent assay kit from R&D System (Minneapolis, MN), and production of nitric oxide (NO) was determined by measuring the accumulated levels of nitrite in the supernatant with the Griess reagent, as described previously [39].

### 2.8. Assay of intracellular ROS

5-(and -6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2-DCFDA) (Molecular Probes), a chloromethyl derivative of H2-DCFDA, passively diffuses into cells in which it is hydrolyzed by intracellular esterases to liberate 2′-7′-dichlorofluorescein (DCF) which, during reaction with oxidizing species, yields a highly fluorescent compound that is trapped inside the cell [37]. Microglia were seeded at $1\times10^5$/well in 96-well plates for one day followed by treatment with 0.6 or 1.2 mM VPA for 24 h. After washing two times with warm Hank's balanced salt solution (HBSS), CM-H2-DCFDA, diluted to a final concentration of 1 μM in phenol red-free HBSS, was added to cultures and incubated for 30 min at 37 °C. Then cultures were added with 0.6 or 1.2 mM VPA in HBSS again for 30 min and followed the treatment with 100 ng/ml LPS for 2 h at 37 °C, fluorescence intensity was measured at 485 nm for excitation and 530 nm for emission using a SpectraMax Gemini XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

### 2.9. Determination of microglia cell number

Primary microglia-enriched culture seeded for one day was treated with indicated concentrations of VPA (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, or 1.2 mM) for 48 h, or with 0.6 mM VPA for different time points (6, 12, 24 or 48 h). After treatment with VPA and vehicle for 48 h, the number and morphology of microglia in cultures were observed under an inverted microscope (Nikon, Tokyo, Japan) at 100×. The total number of microglia was counted by using the CyQUANT cell proliferation assay kit (Molecular Probes).

### 2.10. Statistical analysis

The data were presented as the mean±S.E.M. For multiple comparisons of groups, ANOVA was used. Statistical significance between groups was assessed by paired or unpaired Student's *t*-test, with Bonferroni's correction. A value of $p<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effect of VPA on LPS-induced degeneration of DA neurons

Mesencephalic neuron–glia cultures were pretreated with VPA for 48 h and then stimulated with LPS for 72 h. The degeneration of DA neurons was then determined by [$^3$H] DA uptake assay and counting the number of TH-ir neurons. The [$^3$H] DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up DA to approximately 50% of the vehicle control and this LPS-induced reduction was concentration-dependent prevented by VPA pretreatment (Fig. 1A). At 0.6 mM VPA, which is within the therapeutic range of this drug, the LPS-induced decrease in DA uptake was completely restored and VPA alone at this concentration did not affect DA uptake levels in the cultures. Similar results were obtained when counting the number of TH-ir neurons after immunostaining (Fig. 1B). Thus, LPS-induced loss of TH-ir neurons was prevented by VPA pretreatment in a concentration-dependent manner with a significant effect at 0.2, 0.4 and 0.6 mM. Morphological inspection revealed that LPS treatment not only decreased the number of TH-ir neurons, but also caused a loss of neuronal process (Fig. 2A and B). These characteristics were reversed by VPA pretreatment in a concentration-dependent manner (Fig. 2D–F). VPA at 0.6 mM, either alone or in conjunction with LPS, enhanced the TH immunostaining in both the cell bodies and neuronal processes compared with the vehicle control (Fig. 2C and F).

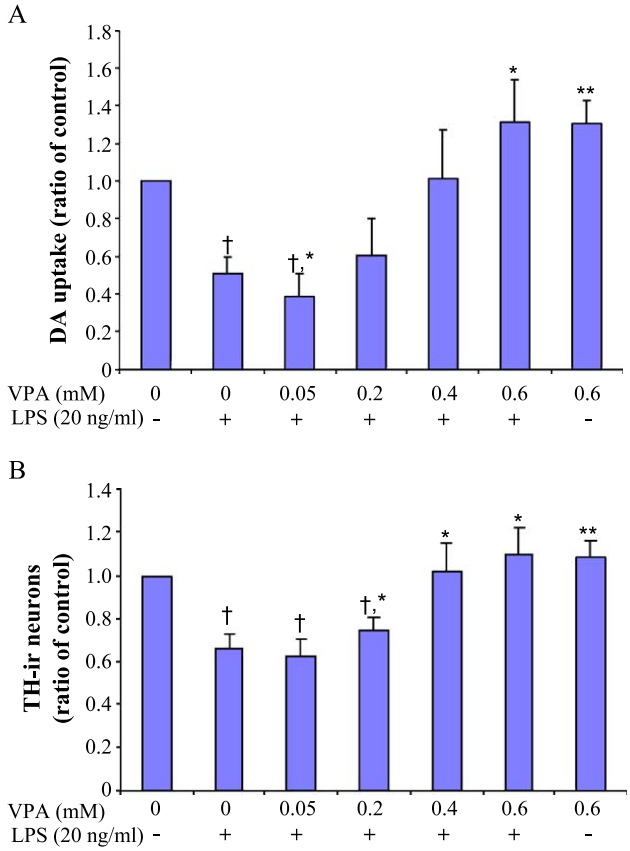


Fig. 1. VPA pretreatment concentration-dependently protects DA neurons from LPS-induced neurotoxicity in rat primary mesencephalic neuron–glia cultures. Experimental conditions are as described in Materials and methods. Briefly, the primary midbrain cultures were pretreated with the indicated concentrations of VPA for 48 h and then treated with 20 ng/ml LPS for 72 h. The analysis of [$^3$H] DA uptake (A) and counting of the number of TH-ir neurons (B) were then performed. Data are expressed as means±S.E.M. from four independent experiments. *$p$<0.05, **$p$<0.01 compared with LPS-treated cultures; †$p$<0.05, ‡$p$<0.01, compared with untreated control.

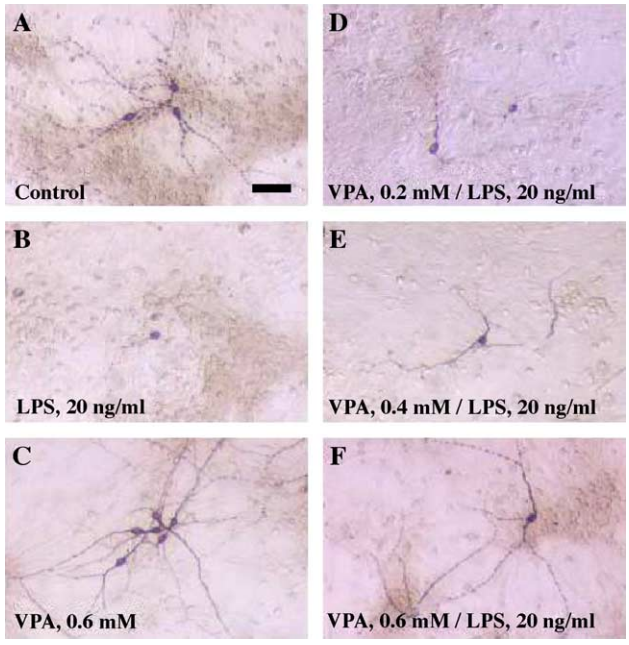


Fig. 2. VPA pretreatment blocks LPS-induced morphological changes of mesencephalic DA neurons immunostained with anti-TH antibody. Experimental conditions are as described in the legend in Fig. 1B. After immunostaining, the images were recorded with an inverted microscope connected to a charge-coupled device camera. Note that LPS-induced loss of cell bodies and neuronal processes were restored by VPA pretreatment. Scale bar, 25 μm.

### *3.2. VPA pretreatment suppresses LPS-induced activation of microglia and production of pro-inflammatory factors in neuron–glia cultures*

Mesencephalic neuron–glia cultures treated with LPS displayed the characteristics of activated microglia such as, increased cell size, irregular shape, and intensified OX-42 immunoreactivity, a specific marker for rat microglia (Fig. 3A,B). The LPS-stimulated activation of microglia was suppressed in neuron–glia cultures pretreated for 48 h with 0.4 or 0.6 mM VPA (Fig. 3D–F). VPA alone did not show significant effects on microglia activation (Fig. 3C).

Activation of microglia mediates the LPS-induced DA neurodegeneration [1,5,15] and this process has been attributed, at least in part, to secretion of a variety of pro-inflammatory and neurotoxic factors, such as TNFα, NO, and superoxide [15,16,33,40,42,46]. As shown in Fig. 4A, pretreatment with 0.4 or 0.6 mM VPA completely blocked LPS-induced production of TNFα in neuron–glia cultures determined at 3 h after LPS stimulation. Even at 0.2 mM

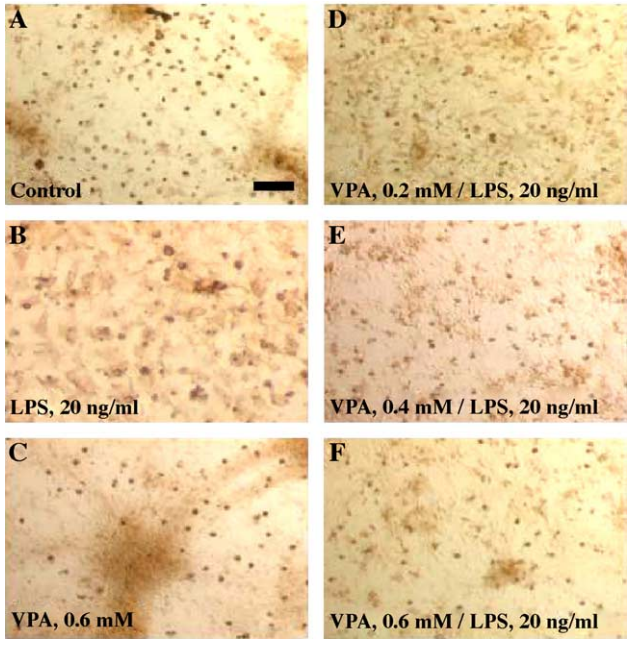


Fig. 3. VPA pretreatment suppresses LPS-induced microglia activation revealed by OX-42 immunostaining. Mesencephalic neuron–glia cultures were pretreated with VPA for 48 h and then treated with 20 ng/ml LPS for 72 h. Immunostaining with an antibody against OX-42 was then performed. Note that microglia activation was indicated by increased cell size, irregular shape and intensified OX-42 staining. These aspects of morphological changes were alleviated by pretreatment with 0.4 or 0.6 mM VPA. Scale bar, 100 μm. The images shown are representative of three independent experiments.

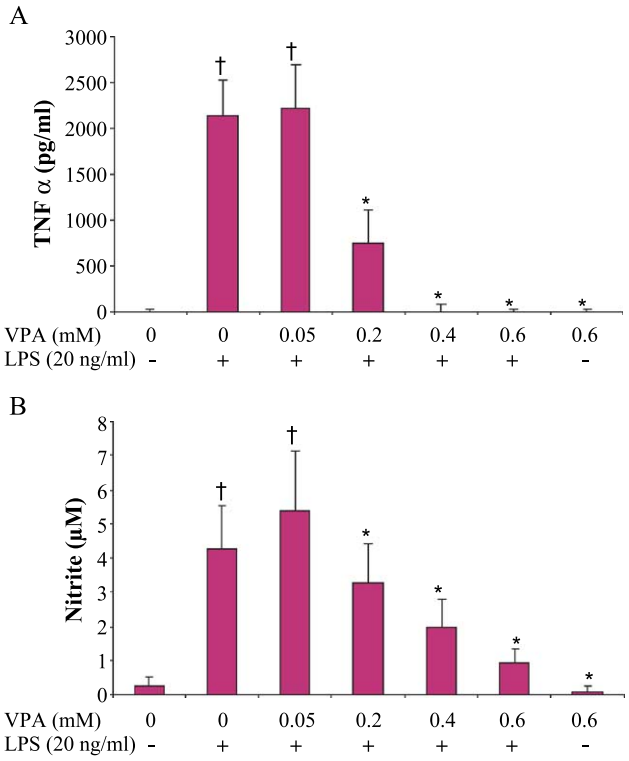


Fig. 4. VPA pretreatment suppresses LPS-induced release of pro-inflammatory factors from rat primary midbrain cultures. Mesencephalic neuron–glia cultures were pretreated for 48 h prior to stimulation with 20 ng/ml LPS. The release of TNF-α was determined 3 h after LPS treatment as described in Materials and methods (A). Levels of nitrite in the supernatant, an indicator of NO production, were determined at 24 h post-LPS treatment (B). Results are means±S.E.M. of four independent experiments. *$p$<0.05 compared with LPS-treated cultures; †$p$<0.05, compared with untreated control.

VPA, LPS-induced TNFα production was also significant inhibited. Accumulation of nitrite, an indicator of LPS-stimulated production of NO, was determined 24 after LPS stimulation. As shown in Fig. 4B, pretreatment with 0.4 and 0.6 mM VPA reduced LPS-stimulated NO production 54% and 78% of the control, respectively.

### *3.3. VPA pretreatment inhibits LPS-induced intracellular reactive oxygen species production in enriched microglia*

To determine if the neuroprotective effect of VPA is due to a reduction in LPS-induced oxidative stress, the level of intracellular reactive oxygen species (iROS) was measured via DCF oxidation in enriched microglia cultures. The iROS level was significantly increased by LPS treatment and this increase was completely blocked by pretreatment with VPA at 0.6 or 1.2 mM (Fig. 5). VPA alone at 0.6 mM, but not 1.2 mM, also reduced basal iROS levels.

### *3.4. VPA treatment decreases the number of microglia*

Primary rat microglia-enriched cultures were used to determine if VPA treatment affected the total number of

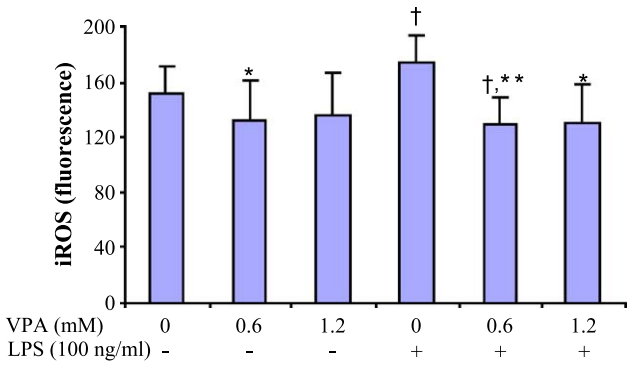


Fig. 5. VPA pretreatment suppresses LPS-induced release of intracellular ROS from rat primary microglia-enriched cultures. Rat microglia-enriched cultures 2 days after plating were pretreated with vehicle or indicated concentrations of VPA for 24 h prior to LPS (100 ng/ml) stimulation. Levels of iROS in enriched microglia were determined by DCFDA at 2 h after LPS treatment. Data are the means±S.E.M. of six experiments. *$p$<0.05, **$p$<0.01 compared with LPS-treated cultures; †$p$<0.05 compared with untreated control.

microglia. Microscopic examination showed that 0.6 mM VPA time-dependently decreased the number of microglia with a significant effect at 24 h and 48 h after treatment

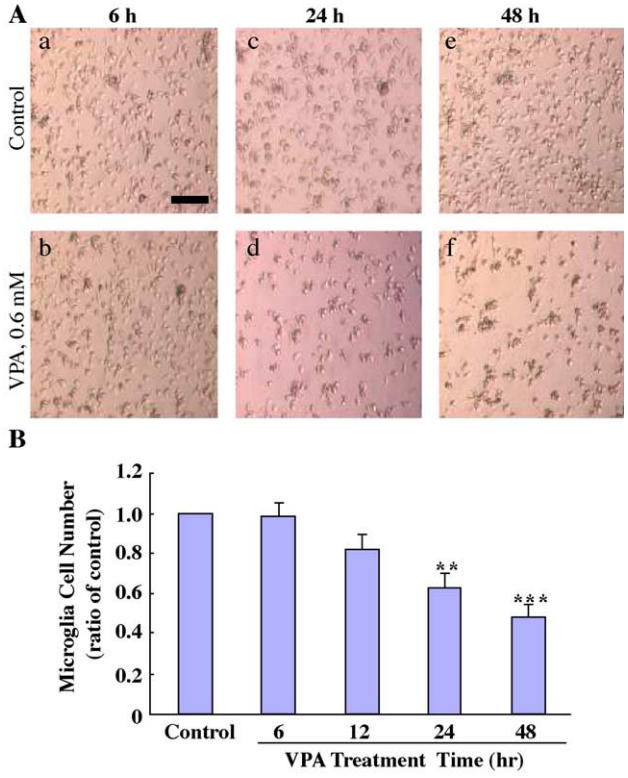


Fig. 6. VPA treatment decreases the number of microglia in a time-dependent manner. Rat microglia-enriched cultures 1 day after plating were treated with 0.6 mM VPA for the indicated times. The morphology of vehicle or VPA-treated microglia cultures were examined (A) and the number of surviving microglia were quantified (B). Data are the means±S.E.M. of three independent experiments performed in triplicate. **$p$<0.01; ***$p$<0.001 compared with the corresponding vehicle-treated controls. Scale bar, 100 μm.

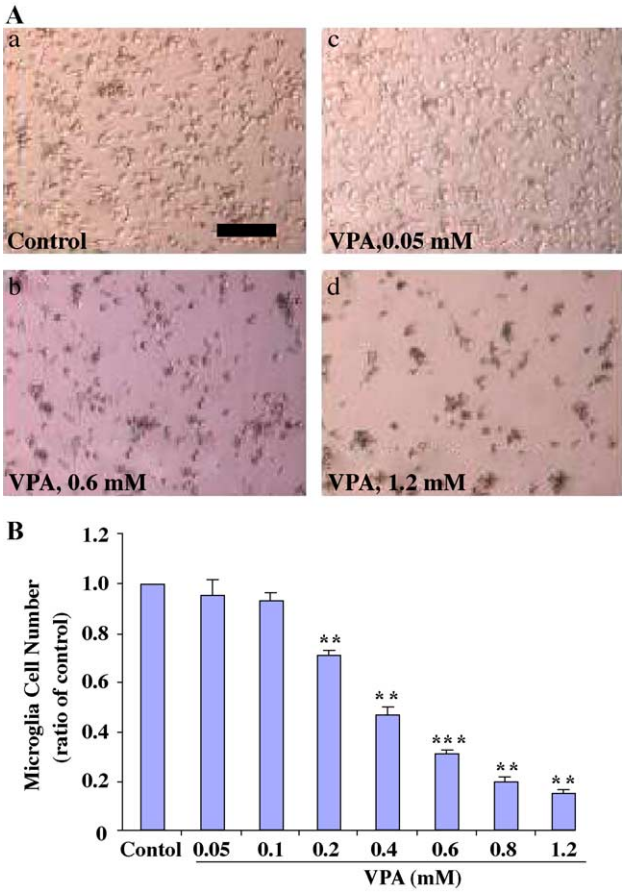


Fig. 7. VPA treatment decreases the number of microglia in a concentration-dependent manner. Rat microglia-enriched cultures 1 day after plating were treated with the indicated concentrations of VPA for 48 h. The morphology of microglia cultures were examined (A) and the number of surviving microglia were quantified (B). Data are the means±S.E.M. of three independent experiments performed in triplicate. $^{**}p<0.01$; $^{***}p<0.001$ compared with the vehicle-treated control. Scale bar, 100 μm.

(Fig. 6A and B). The VPA effect was also concentration-dependent with a robust decrease in microglia number in the concentration range of 0.2–1.2 mM after 48 h treatment (Fig. 7A and B). The loss of microglia was about 80% by treatment with 0.8 mM VPA. Moreover, VPA-induced microglia loss was time and concentration-dependent.

## 4. Discussion

The present study shows that pretreatment of rat mesencephalic neuron–glia cultures with VPA caused a concentration and time-dependent protection of DA neurons from LPS-induced neurotoxicity. These neuroprotective effects of VPA were closely correlated with suppression of microglia activation, as measured by the inhibition of LPS-induced secretion of TNFα and production of NO and iROS. Further, the inhibition of microglia activation was associated with a decrease in the number of microglia. Our results are compatible with the view that VPA protection of midbrain DA neurons from LPS-induced neurotoxicity is due, at least in part, to inhibition of microglia activation, and that the latter effect is likely contributed by a decrease in the number of microglia. Growing evidence strongly suggests that LPS-induced degeneration of DA neurons in midbrain primary cultures is a valid model for screening novel therapeutic agents for Parkinson's disease (for review, see Ref. [32]). The neuroprotective effects of VPA require extended pretreatment and occur within the therapeutic concentration range (i.e. 0.3–1.0 mM) of this drug for treating seizures and bipolar mood disorder. Our results therefore add credibility to the notion that VPA may have additional utility for the therapy of Parkinsonism and other neurodegenerative diseases that involve microglia-mediated inflammation.

It should be noted that VPA is much more robust and potent in blocking LPS-induced TNFα secretion than in inhibiting NO and iROS. Thus, VPA completely blocked LPS-induced TNFα increase, compared with modest effects on other parameters. The effects on TNFα occurred not only at the therapeutic concentrations (i.e. 0.4 and 0.6 mM) but also in the subtherapeutic concentrations (i.e. 0.2 mM) (Fig. 4A). Thus, it is conceivable that VPA blocks TNFα release by decreasing microglia number as well as inhibiting microglia activation through undefined mechanism(s). The anti-inflammatory effects of VPA differ from those of naloxone and dextromethorphan which appear to suppress microglia activation with no apparent effect on microglia number [33,40]. The mechanism underlying VPA-induced loss of microglia is unclear. However, our preliminary data suggest that VPA treatment causes apoptosis of microglia. The requirement of protracted VPA treatment may also suggest an involvement of gene expression. It has been demonstrated that VPA is a direct inhibitor of histone deacetylase (HDAC) by binding to the active site of the enzyme [17,45]. Inhibition of HDAC activation may have profound effects on gene expression (for review, [7]). A recent study shows that in human acute leukemia cells, a HDAC inhibitor, apicidin, induces apoptosis through selective induction of the Fas/Fas ligand, resulting in cytochrome *c* release and caspase activation [27]. Whether VPA induces apoptosis of microglia by such a mechanism resulting from HDAC inhibition awaits future investigation.

TNFα plays a prominent role in inflammatory and immunological responses as well as developmental processes [41–45]. Previous reports show that levels of TNFα receptors on circulating T-lymphocytes and TNFα in postmortem brain and cerebrospinal fluid of Parkinson's patients are significantly increased [45–48]. Additionally, it has been demonstrated that exposure of rat midbrain cultures to TNFα results in a selective loss of DA neurons [31]. Therefore, it seems likely that VPA-induced blockade of TNFα has a central role in protecting against neurotoxicity of LPS. Recent studies have also suggested that VPA elicits neuroprotective effects by direct actions on neurons. These actions include activation of the cell survival

factor Akt [11] and the ERK signaling pathway leading to Bcl-2 expression [12], induction of neurotrophic and neuroprotective proteins such as BDNF [13], GRP 78 [4] and heat shock protein 70 [47], as well as suppression of nuclear accumulation of pro-apoptotic GAPDH [24]. Further studies will be necessary to elucidate whether these mechanisms in addition to anti-inflammatory effects participate in the neuroprotection of mesencephalic DA neurons from LPS-induced neurotoxicity.

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